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Selective antagonism of the AT_1 receptor inhibits angiotensin II stimulated DNA and protein synthesis in primary cultures of human proximal tubular cells

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Selective antagonism of the AT_1 receptor inhibits angiotensin II stimulated DNA and protein synthesis in primary cultures of human proximal tubular cells. The hypertrophy of renal proximal tubular cells occurs as an adaptive response to a variety of stimuli and may be involved with the progression of renal disease. Angiotensin II acting alone or in combination with other growth factors has been implicated in this process. The aims of this study were to identify the role of both angiotensin II and the angiotensin receptor subtypes in DNA synthesis and protein synthesis in human renal proximal tubular cells. Primary cultures of human renal proximal tubular cells were incubated with angiotensin II (10^{-10} M, 10^{-8} M, 10^{-6} M) for 24 to 120 hours either alone or in combination with losartan, PD123319 or 8-bromo-cAMP. Incubation of human proximal tubular cells with angiotensin II (10^{-10} M, 10^{-8} M) induced a significant early increase in [^{3}H]thymidine uptake by 19% and 56% ($P < 0.01$), respectively, and a later increase in total protein content by 30% ($P < 0.01$). The effect of angiotensin II upon DNA and protein synthesis was inhibited by 8-bromo-cAMP and losartan but not by PD123319, indicating that the responses are mediated via the AT_1 receptor and dependent upon the inhibition of adenylate cyclase.

The hypertrophy of the renal tubulointerstitium in renal diseases such as glomerulonephritis indicates a poor prognosis for recovery, often resulting in interstitial fibrosis and end-stage renal failure [1, 2]. While it is clear that hypertrophy of glomerular cells represents a poor prognostic indicator in a variety of renal disease states [3], less is known about the role of compensatory hypertrophy of the proximal tubule cell (PTC). It has been suggested, however, that such PTC hypertrophy may also be a maladaptive process and play a pivotal role in renal disease progression [2]. Although the cellular mechanisms involved in the pathological alteration of tubular growth have not been fully elucidated it is clear that vasoactive peptides such as angiotensin II (Ang II) are probably involved [4].

While the homeostatic role of Ang II is now well established [5], less is known about its potent growth regulatory functions [6–9]. Ang II has been reported to have growth factor-like actions in a number of renal cells [10–13] and to stimulate the transcription and synthesis of type IV collagen, an integral constituent of the basement membrane [14] and a necessary prelude to Ang II induced hypertrophy [10].

Studies *in vivo* serve to reinforce these findings. Ang II infusion into rats has been reported to produce tubular hypertrophy, an increase in type IV collagen deposition [15] and an increase in the transcription of the growth related proto-oncogenes *c-fos* and *Egr-1* [16]. The place of angiotensin converting enzyme (ACE) inhibitors such as captopril in the treatment and amelioration of human diabetic nephropathy is now well established [17], but ACE inhibitors and selective Ang II antagonists have also been reported to ameliorate or shorten the course of renal disease in animal models of interstitial fibrosis and glomerulosclerosis [18–20]. It has been suggested that the growth-promoting effects of Ang II may result from autocrine or paracrine activation of cytokines such as platelet-derived growth factor (PDGF) or transforming growth factor-beta (TGF-B) [21], however, the exact mechanisms involved in altered tubular growth are unclear.

The aim of this study was to determine whether Ang II could stimulate cellular DNA and protein synthesis in primary cultures of human proximal tubular (HPT) cells, and, if so, which angiotensin receptor subtype was involved.

METHODS

Isolation and culture of HPT cells

HPT cells were prepared as previously described [22], from the unaffected pole of kidneys removed at nephrectomy. Briefly, 10 to 15 g of macroscopically normal cortex was chopped and washed in Hank's buffer after which the tissue was digested for one hour at 37°C in modified Hank's buffer containing 0.32 U/ml collagenase A (Boehringer Mannheim, Sussex, UK). The resultant suspension was then filtered through a 75 μm filter, pelleted and washed in Hank's buffer. The cell mixture was then resuspended in Percoll buffer solution (Percoll; Sigma Chemical Co., Dorset, UK) with a starting density of 1.044 g/ml and centrifuged at 13,000 r.p.m. (20,000 g) at 4°C for 30 minutes. After centrifugation three bands were obtained at densities 1.04, 1.06 and 1.065 g/ml. Cells from the 1.06 g/ml band were removed, pelleted and resuspended in ice-cold Dulbecco's Modified Eagle's Medium/Ham's F12 nutritional supplement (DMEM/Ham's F12 1:1 vol/vol; Gibco BRL,

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Scotland, UK). An aliquot of cell suspension was removed for determination of cell number and viability using the Trypan blue exclusion method.

Cell culture

HPT cells were seeded onto 3.5 cm plastic plates (Costar, Buckinghamshire, UK) at a seeding density of 1×10^6 cells/plate (1×10^5 cells/cm^2) and allowed to attach overnight in serum-supplemented medium. The cells were cultured in DMEM/Ham's F12 containing D-glucose (17.5 mmol/liter), sodium bicarbonate (12.5 mmol/liter) supplemented with 10% fetal calf serum (FCS, Gibco), 50 IU/ml penicillin (Gibco) and 50 μg/ml streptomycin (Gibco). Cultures were maintained in an incubator at 37°C within a humidified atmosphere of 95% air/5% CO_2 with medium changed every 48 hours.

Incubation with Ang II, losartan, PD123319 and 8-bromo-cAMP

After a further 48 hour period, medium was replaced by DMEM/Ham's F12 containing 10% FCS in the presence or absence of Ang II (10^{-10}, 10^{-8} or 10^{-6} M; Sigma).

Because consistently significant effects upon DNA and protein synthesis, compared to baseline were observed following incubation with Ang II 10^{-8} M and at days 3 to 5 and 7 to 10 post-seeding, respectively, this concentration of Ang II and the time points at days 5, 7, 10 were chosen for all further experiments using the AT_1 and AT_2 antagonists.

HPT cells were incubated with losartan (10^{-6} M; DuPont, Detroit, MI, USA), or PD123319 (10^{-6} M; Parke-Davis, Detroit, MI, USA), or 8-bromo-cAMP (10^{-6} M, Sigma) either in the presence or absence of Ang II (10^{-8} M). Medium was changed every two days.

Measurement of cellular DNA synthesis

Cellular DNA synthesis was estimated using [^{3}H]thymidine (26 Ci/mmol; Amersham International plc, Little Chalfont, Bucks, UK) incorporation into cell DNA. Cultures were pulsed for one hour with 1 μCi/ml [^{3}H]thymidine at 37°C in a humidified 95% air/5% CO_2 atmosphere after which they were washed twice with ice-cold phosphate buffered saline (PBS). The cells were then left for 30 minutes in 1 ml ice-cold perchloric acid (0.2 M), after which they were again washed twice with PBS before being scraped into 1 ml NaOH (0.3 M). Radioactivity in samples was determined using liquid scintillation counting and measured on a Packard Tri-Carb Liquid Scintillation Analyser (Canberra Packard, Pangbourne, Berkshire, UK) with external standard quench correction.

Measurement of RNA synthesis

Total RNA synthesis was estimated using [^{3}H]uridine (26 Ci/mmol; Amersham International plc, Little Chalfont, Bucks, UK) incorporation into cell RNA. [^{3}H]uridine was added to growth media to produce a final concentration of 1 μCi/ml and the cultures were incubated for 24 hours at 37°C in a humidified 95% air/5% CO_2 atmosphere. On completion of incubation, the cultures were washed twice with ice-cold PBS and then scraped into 1 ml NaOH (0.3 M). The radioactivity content of samples was measured as described above.

Measurement of de novo protein synthesis

Incorporation of [^{3}H]leucine (158 Ci/mmol; Amersham International plc) was used as a sensitive measure of *de novo* protein synthesis. After addition of [^{3}H]leucine to the growth media to produce a final concentration of 5 μCi/ml, the cultures were incubated for 24 hours at 37°C in a humidified 95% air/5% CO_2 atmosphere. Cultures were then washed twice with ice-cold PBS after which 1 ml ice-cold trichloroacetic acid solution (10% w/v) was added. Following 10 minutes on ice, this step was repeated twice. After another two washes in PBS, the cell residue left on the plate was solubilized in 1 ml NaOH (0.3 M). The radioactive content of samples was determined as described above.

Measurement of total protein content

The total protein content of samples was measured using a modification of the Lowry assay [23] using bovine serum albumin to produce a standard curve.

Presentation of data and statistical analysis

Results obtained from the [^{3}H]thymidine incorporation were expressed as dpm/mg protein/hour, [^{3}H]uridine and [^{3}H]leucine assays results were expressed as dpm/mg protein/24 hours. The total protein content of cultures was expressed as mg protein/plate. The data are presented as mean ± SD. Statistical significance was determined using a one way ANOVA. A P value of less than 0.05 was considered to indicate significance.

RESULTS

HPT cell characterization

Primary cultures of HPT cells grew to form confluent monolayers over seven days and exhibited the cobblestone morphology characteristic of epithelial cells. The cultures maintained their epithelial appearance throughout the duration of the experiments with no significant evidence of fibroblast contamination or overgrowth. We have previously reported that HPT cultures prepared and grown in this manner maintain phlorizin-inhibitable Na^+-dependent D-glucose transport [24], anion and cation transport mechanisms and a significant cAMP response to parathyroid hormone (10^{-6} M) with no similar response to vasopressin (10^{-6} M) or calcitonin (10^{-6} M). Prior to use HPT cells were also characterized according to their immunohistochemical and renal enzyme histochemical staining as previously described [25].

Effect of angiotensin II on HPT cell cultures

Incubation of HPT cell cultures with Ang II 10^{-10} and 10^{-8} M produced a significant increase in [^{3}H]thymidine uptake of 19% and 42%, respectively ($621 \pm 39 \times 10^3$ to $736 \pm 40 \times 10^3$ [10^{-10} M], $882 \pm 72 \times 10^3$ [10^{-8} M], dpm/mg protein/hour, $N = 4$, $P < 0.01$), compared with control (Fig. 1A). The maximal effect was observed at day 5 of incubation.

After seven days in culture, the cells attained confluence. From this time point onwards, Ang II stimulated a significant increase in total cell protein content (Fig. 1B), RNA synthesis measured as [^{3}H]uridine uptake (Fig. 1C) and protein synthesis measured as [^{3}H]leucine uptake (Fig. 1D). After 10 days incubation with Ang II (10^{-8} and 10^{-6} M) total protein content increased by 20% and 22%, respectively [0.860 ± 0.06 to 1.035 ± 0.02 (10^{-8} M) and 1.050 ± 0.03 (10^{-6} M) mg protein/plate, $N = 4$, $P < 0.01$), compared with control. Because the maximal effect on DNA and

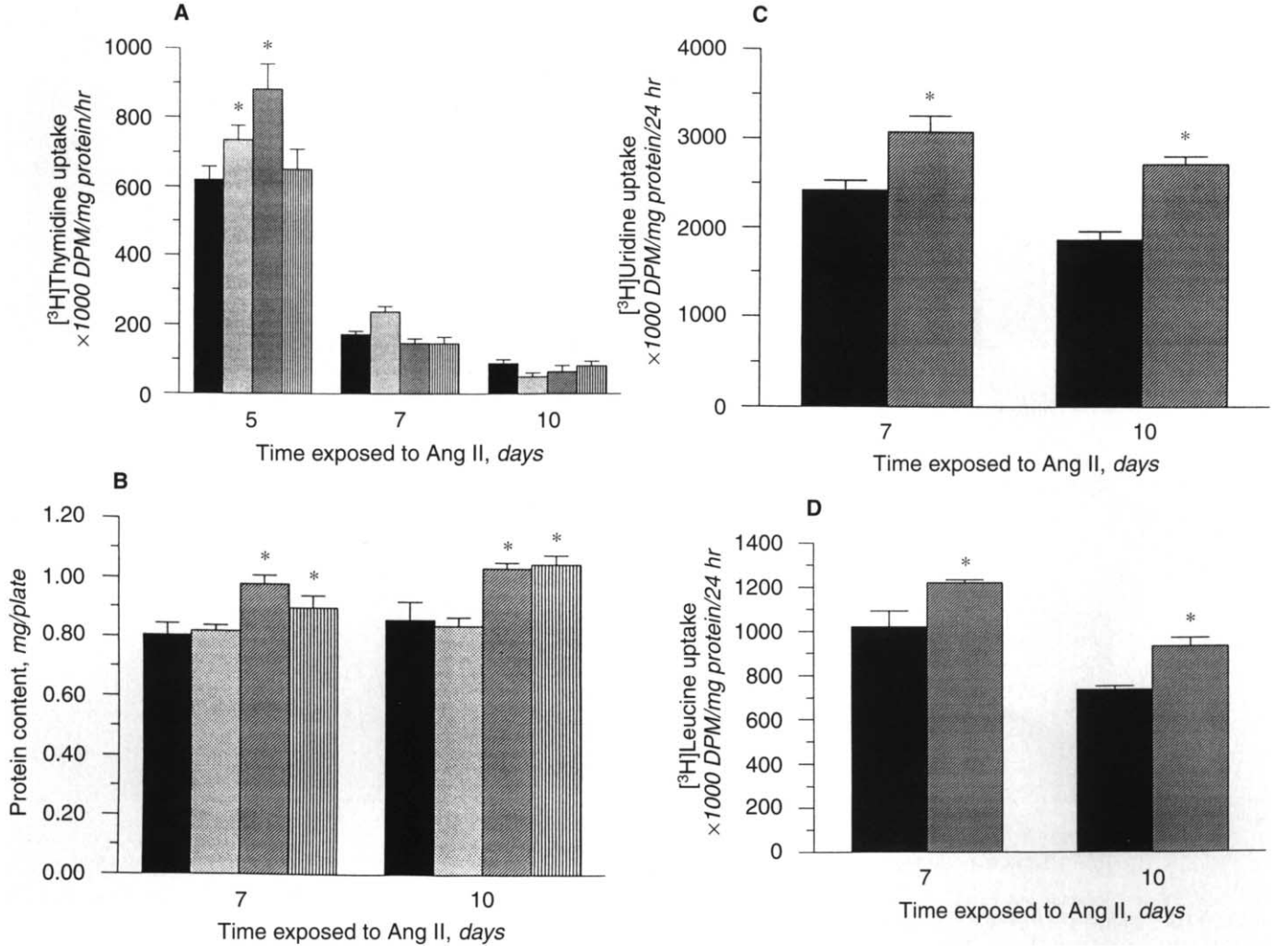

Fig. 1. *A.* [^{3}H]thymidine uptake by cultures of HPT cells after 5, 7 and 10 days exposure to Ang II (10^{-10}, 10^{-8} and 10^{-6} M) ($N = 4$). (*B*) The total protein content of HPT cells after 7 and 10 days expose to Ang II (▨ 10^{-10}, ▨ 10^{-8}, and ▨ 10^{-6} M; $N = 4$; $P < 0.01$ vs. ■ control). (*C*) The effect of Ang II (10^{-8} M) upon [^{3}H]uridine incorporation into HPT cells following 7 and 10 days incubation ($N = 4$); symbol (■) is control. (*D*) The effect of Ang II (▨ 10^{-8} M) upon [^{3}H]leucine incorporation into HPT cells following 7 and 10 days incubation ($N = 4$; $P < 0.01$ vs. ■ control).

protein synthesis was observed for cells incubated with Ang II 10^{-8} M all further experiments were carried out with this concentration of Ang II. Incubation with Ang II 10^{-8} M also produced a significant increase in [^{3}H]uridine uptake by 45% (1872 ± 96 × 10^3 to 2712 ± 96 × 10^3 (10^{-8} M) dpm/mg protein/24 hr, $N = 4$, $P < 0.01$] and [^{3}H]leucine uptake by 26% (737 ± 17 × 10^3 to 931 ± 38 × 10^3 [10^{-8} M] dpm/mg protein/24 hr, $N = 4$, $P < 0.01$) at day 10.

Effect of losartan and PD123319 upon angiotensin II-stimulated HPT cell cultures

By day 5, incubation with losartan produced a significant 29% inhibition of Ang II-stimulated DNA synthesis returning levels to control values [882 ± 72 × 10^3 (Ang II 10^{-8} M) to 626 ± 93 × 10^3 dpm/mg protein/hr (Ang II 10^{-8} M + losartan 10^{-6} M, $N = 4$, $P < 0.01$; Fig. 2A]. At day 10 losartan also produced a significant 20% inhibition of Ang II-stimulated total protein content (1.04 ± 0.02 to 0.83 ± 0.09 mg protein/plate, $N = 4$, $P < 0.01$; Fig. 2B), 33% reduction in [^{3}H]uridine uptake (2712 ± 96 × 10^3 to 1824 ± 72 × 10^3 dpm/mg protein/24 hr, $N = 4$, $P < 0.01$; Fig. 2C) and a 23% reduction in [^{3}H]leucine uptake (931 ± 38 × 10^3 to 721 ± 5 × 10^3 dpm/mg protein/24 hr, $N = 4$, $P < 0.01$; Fig. 2D). Incubation with losartan completely blocked the effects of Ang II upon DNA and protein synthesis. Incubation with PD123319 had no effect on Ang II-stimulated DNA, RNA or protein synthesis.

Effect of 8-bromo-cAMP upon angiotensin II-stimulated DNA and protein synthesis

Incubation with 8-bromo-cAMP (10^{-6} M) produced similar effects to those observed with losartan returning all values following incubation with Ang II to baseline. 8-Bromo-cAMP produced a significant 29% inhibition of Ang II-stimulated [^{3}H]thymidine uptake (882 ± 72 × 10^3 to 625 ± 50 × 10^3 dpm/mg protein/hr, $N = 4$, $P < 0.01$; Fig. 3A) of total protein content by 26% (1.04 ± 0.02 to 0.78 ± 0.06 mg protein/plate, $N = 4$, $P < 0.01$; Fig. 3B), of [^{3}H]uridine uptake by 35% (2712 ± 96 × 10^3 to 1776 ± 48 × 10^3 dpm/mg protein/24 hr, $N = 4$, $P < 0.01$; Fig. 3C)

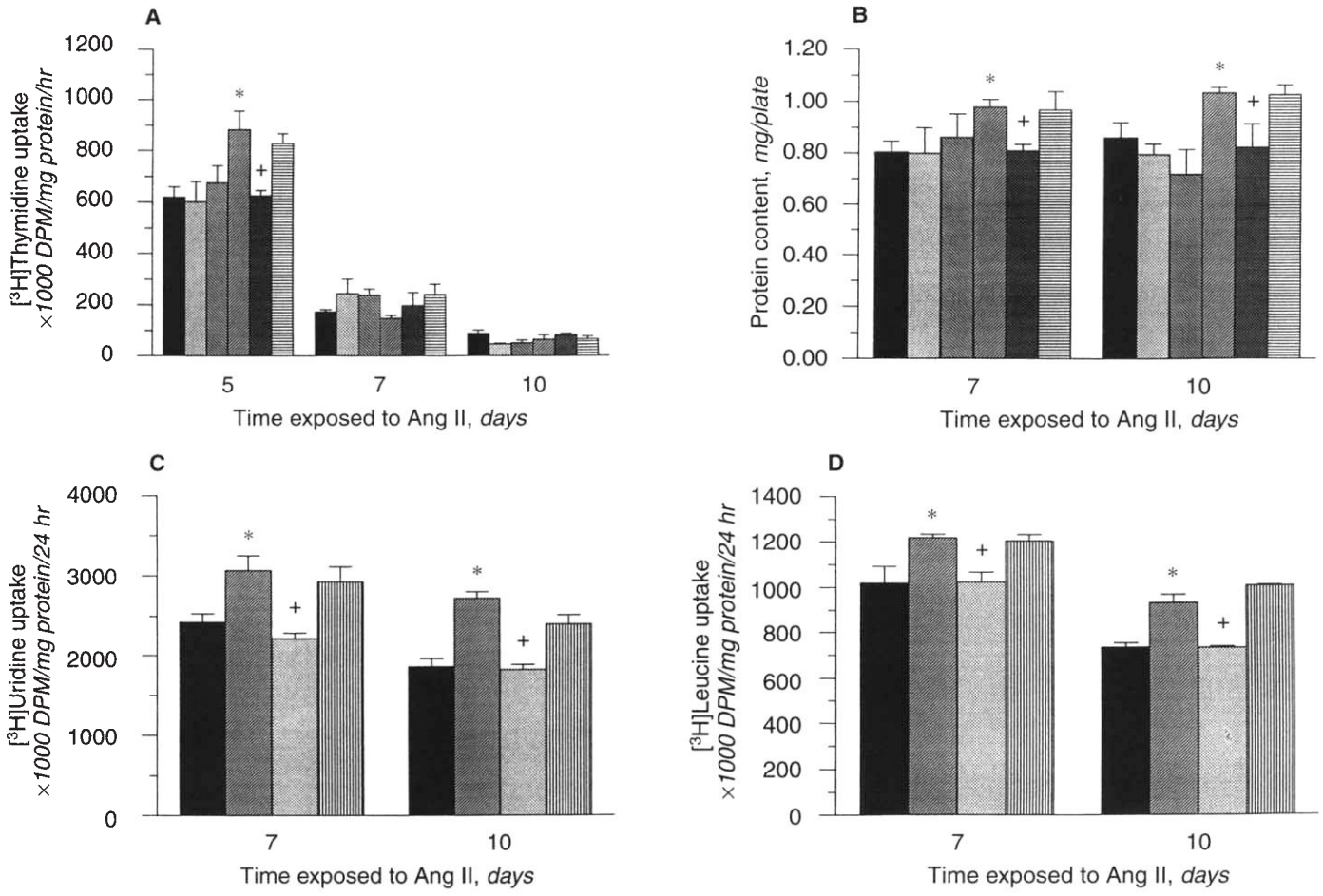

Fig. 2. *A.* **Effect of losartan and PD123319 (both 10^{-6} M) on Ang II-induced thymidine incorporation into HPT cells following 5, 7 and 10 days exposure to Ang II (10^{-8} M; $N = 4$).** (*B*) The effect of losartan and PD123319 (both 10^{-6} M) on total protein content of HPT cells following 7 and 10 days exposure to Ang II (10^{-8} M; $N = 4$; $^*P < 0.01$ vs. control; $+P < 0.01$ vs. 10^{-8} M Ang II). Symbols are: (■) control; (⊡) 10^{-6} M lostartan; (▨) 10^{-6} M PD123319; (▨) 10^{-8} M Ang II; (▩) 10^{-8} M Ang II + 10^{-6} M lostartan; (▤) 10^{-8} M Ang II + 10^{-6} M PD123319. (*C*) The effect of losartan and PD123319 (both 10^{-6} M) on Ang II stimulated [^{3}H]uridine incorporation into HPT cells following 7 and 10 days incubation with Ang II (10^{-8} M; $N = 4$). (*D*) The effect of losartan and PD123319 (both 10^{-6} M) on Ang II stimulated [^{3}H]leucine incorporation into HPT cells following 7 and 10 days incubation with Ang II (10^{-8} M; $N = 4$; $^*P < 0.01$ vs. control; $+P < 0.01$ vs. 10^{-8} M Ang II). Symbols are: (■) control; (▨) 10^{-8} M Ang II; (▩) 10^{-8} M Ang II + 10^{-6} M lostartan; (▤) 10^{-8} M Ang II + 10^{-6} M PD123319.

and [^{3}H]leucine by 28% ($931 \pm 38 \times 10^3$ to $670 \pm 5 \times 10^3$ dpm/mg protein/24 hr, $N = 4$, $P < 0.01$; Fig. 3D).

DISCUSSION

Proliferation and compensatory hypertrophy of PT cells following recovery from acute and chronic renal damage are adaptive responses essential for the re-establishment of the tubule and maintenance of glomerular filtration [26]. It has, however, been suggested that hypertrophy of the PT cell may in part contribute to the progression of chronic renal disease and end-stage renal failure [9]. *In vivo* and *in vitro* studies suggest that Ang II may play an important role in these changes. *In vitro*, Ang II has been reported to stimulate cell hypertrophy in a variety of renal cell lines including the murine SV40 virus-transformed PT cell line [10], porcine LLC-PK$_1$ cell line [11] and murine mesangial cells [12]. *In vivo,* the administration of ACE inhibitors and Ang II receptor antagonists to animal models of glomerulosclerosis and interstitial fibrosis has been reported to ameliorate and foreshorten the disease process [18–20]. Although prospective studies have confirmed the place of ACE inhibitors in the treatment and amelioration of diabetic nephropathy, little is known about the mechanism of action of Ang II or selective Ang II antagonists on the human kidney. Furthermore, all of the *in vivo* and *in vitro* studies have been performed in animal models or non-human or virally transformed immortalized cell lines, which differ considerably in biochemical characteristics from the human PT cell.

The aim of this study was to determine the effects of Ang II on primary cultures of HPT cells that have been previously shown to maintain many of the characteristics and functions of the PT cell *in vivo* and to determine whether such primary cultures of HPT cells may be used as a model for the human situation *in vivo*.

In this series of experiments incubation with Ang II stimulated a significant increase in DNA synthesis (day 5) followed by a significant but later increase in protein synthesis at day 10. The greatest increase in DNA synthesis was observed following incubation with Ang II (10^{-8} M). Ang II (10^{-10} M) also had a

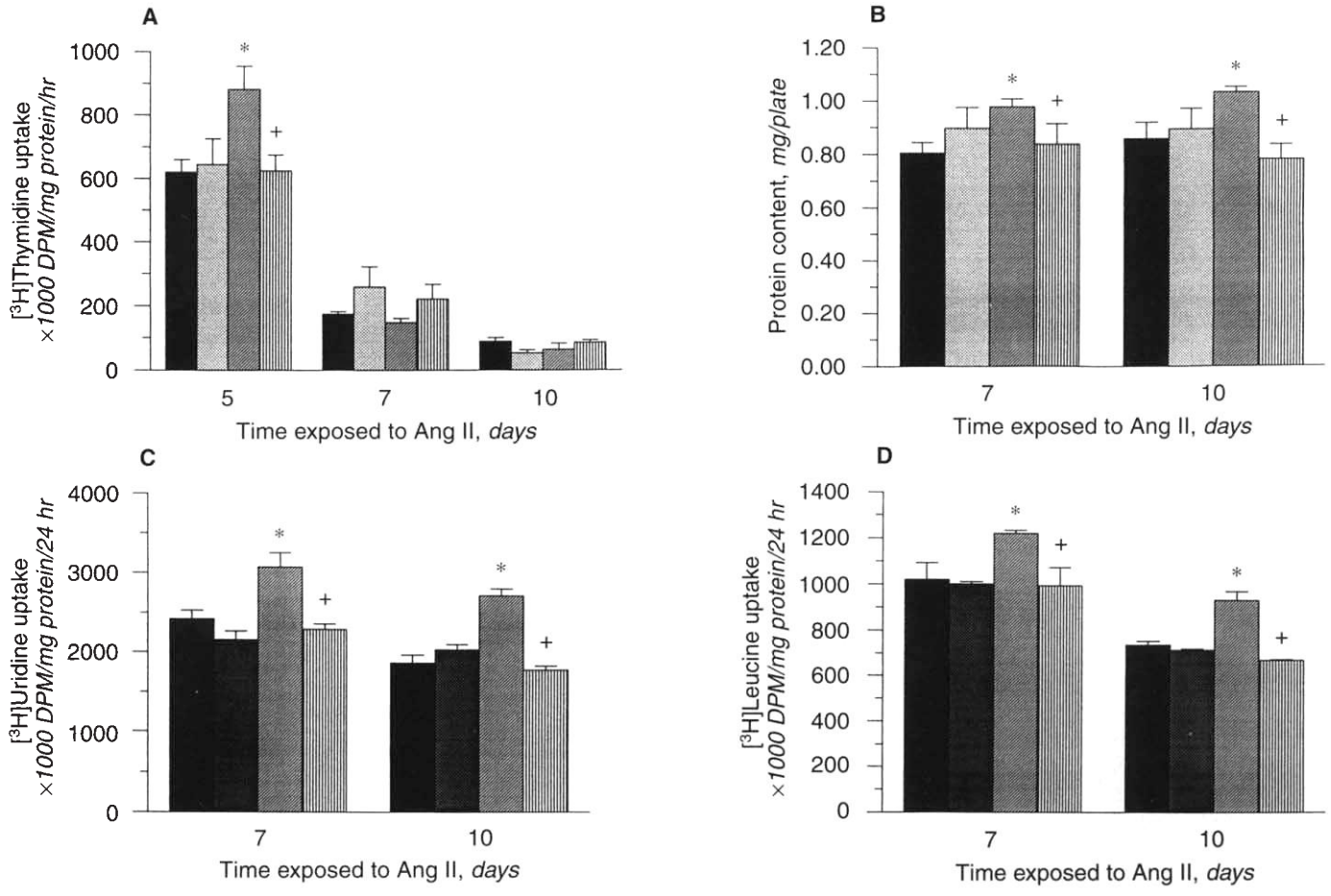

Fig. 3. *A.* **The effect of 8-bromo-cAMP on ANG II induced thymidine incorporation into HPT cells following 5, 7 and 10 days exposure to Ang II (10^{-8} M; $N = 4$; *$P < 0.01$ vs. control; +$P < 0.01$ vs. 10^{-8} M Ang II).** Symbols are: (■) control; (▨) 10^{-6} 8-bromo-cAMP; (▨) 10^{-8} M Ang II; (▤) 10^{-8} M Ang II + 10^{-6} M 8-bromo-cAMP. (*B*) The effect of 8-bromo-cAMP on total protein content of HPT cells following 7 and 10 days exposure to Ang II (10^{-8} M; $N = 4$). (*C*) The effect of 8-bromo-cAMP on Ang II induced uridine incorporation into HPT cells following 7 and 10 days incubation with Ang II (10^{-8} M; $N = 4$). (*D*) The effect of 8-bromo-cAMP on Ang II induced leucine incorporation into HPT cells following 7 and 10 days incubation with Ang II (10^{-8} M) ($N = 4$).

significant but lesser effect, while Ang II (10^{-6} M) had no effect on cell DNA synthesis. Similarly, the greatest increase in protein synthesis was observed following incubation with Ang II (10^{-8} M), although a similar increase was observed following incubation with Ang II 10^{-6} M. Similar concentration dependent effects have been reported for the actions of Ang II on Na^+ transport in the rat PT and rat mesangial cell proliferation [6, 27, 28]. Why the proliferative and hypertrophogenic effects of Ang II should be maximal at a concentration of 10^{-8} M is difficult to determine. There are several possible explanations. It has been reported that the AT_1 receptor is rapidly down-regulated or desensitized in the presence of high saturating levels of Ang II [29]. Sasamura et al have reported that incubation with low concentrations of Ang II (10^{-10} M) produced desensitization of the AT1 receptor via a PKC mediated pathway, while incubation with high doses of Ang II (10^{-7} M) brought about desensitization via a non-PKC mediated effect [30]. Recently the AT_2 receptor has been reported to antagonize the proliferative effects of the AT_1 receptor, stimulation producing inhibition of cell proliferation [30, 31]. Using radioligand binding we have demonstrated the presence of both AT_1 and AT_2 receptors in human proximal tubular cells. Losartan, the AT1-specific ligand blocked 58 ± 4% of Ang II binding sites, while PD123319, the specific AT2 antagonist, blocked 55 ± 7% of binding sites. Competition studies between [^{125}I]Ang II and increasing concentrations of Ang II confirmed the presence of two site binding for Ang II. Further analysis of data using Radlig software confirmed a two-site best fit with a high and a low affinity binding site with K_d values of 0.18 ± 0.07 and 60.6 ± 10.7 and B_{max} values of 0.43 ± 0.10 and 45.7 ± 22.4, respectively. Primary cultures of HPT cells therefore possess both AT_1 and AT_2 receptors. It is therefore possible that the growth inhibitory actions of the AT_2 receptor only become apparent at high "supraphysiological" concentrations of Ang II. Stoll et al have reported that blockade of the AT_2 receptor stimulates proliferation in a rat vascular smooth muscle cell line, however these experiments were performed with relatively high concentrations of Ang II (10^{-7} M) [30]. Again, it is possible that the AT_2 receptor is stimulated at higher concentrations of Ang II. Ang II has also been reported to mediate its effects by two possible pathways, one mediates a decrease in cAMP via a G_i protein linked to adenylate

cyclase, the other stimulates the phosphatidylinositol pathway. It is possible that low concentrations of Ang II activate the proliferative pathway while high concentrations interact with additional inhibitory signal transduction pathways. Both AT_1 and AT_2 receptors are known to be present in PT cells [6, 32], and the AT_2 receptor subtype has been reported to be the predominant subtype in large preglomerular vessels prepared from human kidney and throughout the tubulointerstitium of normal human kidney, representing approximately 60% of total Ang II receptors [33]. The results obtained in this study suggest that both the early Ang II induction of DNA synthesis and later induction of protein synthesis are mediated via the AT_1 receptor. Both responses were inhibited in the presence of losartan, the selective AT_1 antagonist, while PD123319, a selective AT_2 receptor antagonist, failed to have any effect. We have previously reported similar results for rat proximal tubular cells, cultured in serum containing medium [34].

Maximal Ang II stimulation of DNA synthesis was observed at day 5 of culture when cells were growing rapidly in the form of small colonies. By day 7 cells had reached confluence and from this point onwards, Ang II had no effect on [^{3}H]thymidine uptake but produced a significant increase in RNA synthesis, *de novo* protein synthesis and total protein content, suggesting that Ang II was stimulating cellular hypertrophy. Wolf et al previously reported that incubation with Ang II (10^{-8} M) stimulated cell hypertrophy, while inhibiting cell proliferation in an LLC-PK_1 cell line cultured in serum free medium [11]. Unlike Wolf et al our cells were cultured in serum containing medium and were not serum rested. Fetal calf serum contains a wide variety of growth factors [35], and it is now recognized that Ang II-induced proliferation only occurs in the presence of additional growth factors including EGF, PDGF and fibroblast growth factor or under conditions where these factors can be synthesized by the growing cells [36–38]. We have previously reported that FCS is essential for Ang II-induced cellular proliferation to occur in primary cultures of rat proximal tubular cells and it is likely that the same is true for primary cultures of human proximal tubular cells [34].

It is believed that the AT_1 receptor in PT cells is coupled to adenylate cyclase via a G_i protein sensitive to pertussis toxin and that Ang II stimulated hypertrophy is associated with a decrease in intracellular cAMP concentration [10, 11]. In this series of experiments incubation of HPT cells with 8-bromo-cAMP (10^{-6} M) completely abolished the cellular response to Ang II, indicating that the observed Ang II-induced increase in DNA, RNA, *de novo* protein synthesis and total protein content is mediated by the AT_1 receptor and involves down-regulation of adenylate cyclase probably via a G_i protein.

There is now increasing evidence that PT hypertrophy is an adaptive response to many physiological and pathological stimuli and that it is intimately involved in the progression of renal disease. It is also now clear from both *in vivo* and *in vitro* studies that Ang II is a potent growth factor. The results of this study clearly demonstrate that Ang II is a potent stimulator of DNA and protein synthesis in primary cultures of HPT cells, and that both responses are mediated by the AT_1 receptor and fully inhibited by the AT_1 receptor antagonist losartan.

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